

Review

Methods of studying soil microbial diversity

Jennifer L. Kirk^a, Lee A. Beaudette^{a,1}, Miranda Hart^b, Peter Moutoglis^c,
John N. Klironomos^b, Hung Lee^a, Jack T. Trevors^{a,*}

^aDepartment of Environmental Biology, University of Guelph, Ontario Agricultural College, Guelph, Ontario, Canada, N1G 2W1

^bDepartment of Botany, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

^cPremierTech, Rivière-du-Loup, Québec, Canada G5R 6C1

Received 1 December 2003; accepted 7 April 2004

Available online 15 June 2004

Abstract

Soil microorganisms, such as bacteria and fungi, play central roles in soil fertility and promoting plant health. This review examines and compares the various methods used to study microbial diversity in soil.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Arbuscular mycorrhizal fungi (AMF); Bacteria; Biology; Diversity; DNA; Ecology; Fungi; Molecular ecology; Soil

1. Introduction

Soil bacteria and fungi play pivotal roles in various biogeochemical cycles (BGC) (Molin and Molin, 1997; Trevors, 1998b; Wall and Virginia, 1999) and are responsible for the cycling of organic compounds. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (George et al., 1995; Timonen et al., 1996), plant health (Srivastava et al., 1996; Filion et al., 1999; Smith and Goodman, 1999), soil structure (Wright and Upadhyaya, 1998; Dodd et al., 2000) and soil fertility (Yao et al., 2000; O'Donnell et al., 2001).

Our knowledge of soil microbial diversity is limited in part by our inability to study soil microorganisms. Torsvik et al. (1990a,b) estimated that in 1 g of soil there are 4000 different bacterial “genomic units” based on DNA–DNA reassociation. It has also been estimated that about 5000 bacterial species have been described (Pace, 1997, 1999). Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices. It is not known if this 1% is representative of the bacterial population (Torsvik et al., 1998). An estimated 1,500,000 species of fungi exist in the world (Giller et al., 1997). But unlike bacteria, many fungi cannot be cultured by current standard laboratory methods (Thorn, 1997; van Elsas et al., 2000). Although molecular methods have been used to study soil bacterial communities, very little research has been undertaken for soil fungi (van Elsas et al., 2000).

All organisms in the biosphere depend on microbial activity (Pace, 1997). Soil microorganisms are vital for

* Corresponding author. Tel.: +1-519-824-4120x53367; fax: +519-837-0442.

E-mail address: jtrevors@uoguelph.ca (J.T. Trevors).

¹ Current address: Environment Canada, Wastewater Technology Center, Burlington, Ontario, Canada L7R.

the continuing cycling of nutrients and for driving above-ground ecosystems (van der Heijden et al., 1998; Cairney, 2000; Klironomos et al., 2000; Ovreas, 2000). While many anthropogenic activities, such as city development, agriculture, use of pesticides and pollution can potentially affect soil microbial diversity, it is unknown how changes in microbial diversity can influence below-ground and above-ground ecosystems. Before we can address how changes in microbial community structure influences ecosystem functions, there is the need for reliable and accurate mechanisms of studying soil microorganisms. This article will review the current methods, and their advantages and disadvantages, for studying microbial diversity in soil.

2. General limitations in studying microbial diversity

There are problems associated with studying bacterial and fungal diversity in soil. These arise not only from methodological limitations, but also from a lack of taxonomic knowledge. It is difficult to study the diversity of a group of microorganisms when it is not understood how to categorize or identify the species present.

2.1. Spatial heterogeneity

When studying microbial diversity, replicates of 1 to 5 g of soil are often used to measure diversity and then conclusions about the community are made. There are numerous problems with this approach. One is the innate heterogeneity of soil and thus of spatial distribution of the microorganisms (Trevors, 1998b). Franklin and Mills (2003) used multiple spatial scales, with sampling intervals ranging from 2.5 cm to 11 m, to study the spatial heterogeneity of soil microbial communities in an agricultural soil. They reported that microbial communities may have several nested levels of organization, and that they could be dependent on different soil properties or groups of properties. Microbial communities exist on such a small scale, that possibly 1 to 5 g of soil could bias results and favour detection of dominant populations (Grundmann and Gourbiere, 1999). Grundmann and Gourbiere (1999) suggested that sampling of soil has to be done on a smaller scale with more samples to

assess the diversity of microorganisms in the microhabitats in soil. Another problem with this approach is that soil is heterogeneous, containing many microhabitats that are suitable for microbial growth. As a result, bacteria are highly aggregated in soil existing in clumps or “hot spots”. Plants also influence the spatial distribution of soil bacteria (Wall and Virginia, 1999) and fungi, as shown by an approximately two-fold increase in bacterial numbers in the rhizosphere over bulk soil (Curl and Truelove, 1986). Arbuscular mycorrhizal fungi (AMF) require a plant host to survive. Therefore, their distribution in soil is also clustered around plant species.

Much of what is known about soil fungal diversity has resulted from field studies of sporocarps or by morphological descriptions of below-ground fungal structures, especially for mycorrhizal fungi (Horton, 2002). Often, fungal diversity studies using data from above-ground fruiting bodies do not correspond to those using below-ground fungal structures (Horton, 2002). This discrepancy can be caused by the sporadic production of sporocarps as well as the lack of information about below-ground structures.

Very little is known about spatial and temporal variability of microorganisms in soil (Sanders and Fitter, 1992; Johnson et al., 1997; Trevors, 1998b). If researchers sample soil in a traditional random fashion, microbial diversity and population size could be grossly underestimated resulting in high variability between replicates and low statistical power (Klironomos et al., 1999). Klironomos et al. (1999) suggested using a combination of geostatistical analyses to describe spatial distribution of subsurface microorganisms together with power analyses to assess the required sample size. This approach should reduce variability in sampling and provide a more representative sampling regime.

2.2. Inability to culture soil microorganisms

The immense phenotypic and genetic diversity found in soil bacterial and fungal communities makes it one of the most difficult communities to study (Ovreas et al., 1998). It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques (Borneman et al., 1996; Giller et al.,

1997; Pace, 1997; Torsvik et al., 1998; Trevors, 1998b). It is possible this 1% of culturable bacteria is representative of the entire population and that the other 99% are simply in a physiological state that eludes our ability to culture them (Rondon et al., 1999). However, it is also likely that the 99% are phenotypically and genetically different from the 1% and only the minority of the population is represented (Rondon et al., 1999, 2000). Many fungal species also elude culturing in the laboratory (van Elsas et al., 2000). To overcome problems associated with non-culturable bacteria and fungi, various methods have been developed to identify and study these microorganisms including fatty acid analysis and numerous DNA- and RNA-based methods.

2.3. Limitations of molecular-based methods

Molecular techniques based on PCR have been used to overcome the limitations of culture-based methods; however, they are not without their own limitations.

Lysis efficiency of cells or fungal structures varies between and within microbial groups (Prosser, 2002). Bacteria exist in or on the surface of soil aggregates; therefore, the ability to separate these cells from soil components is vital for studying biodiversity (Trevors, 1998a). If the method of cell extraction used is too gentle, Gram-negative, but not Gram-positive bacterial cells would be lysed. If the method is too harsh, both Gram-negative and Gram-positive cells may be lysed but their DNA may become sheared (Wintzingerode et al., 1997). Lysis efficiency also varies for different fungal cells. Spores will lyse differently than mycelia and mycelia of different ages will also have different lysing efficiency (Prosser, 2002). The variation in the ability to break open cells or fungal structures can lead to biases in molecular-based diversity studies.

The method of DNA or RNA extraction used can also bias diversity studies. Harsh extraction methods, such as bead beating, can shear the nucleic acids, leading to problems in subsequent PCR detection (Wintzingerode et al., 1997). Different methods of nucleic acid extractions will result in different yields of product (Wintzingerode et al., 1997). With environmental samples, it is necessary to remove inhibitory substances such as humic acids, which can be co-

extracted and interfere with subsequent PCR analysis. Subsequent purification steps can lead to loss of DNA or RNA, again potentially biasing molecular diversity analysis.

Differential amplification of target genes can also bias PCR-based diversity studies. Typically, 16S rRNA, 18S rRNA or ITS regions are targeted by primers for diversity studies because these genes/fragments are present in all organisms, they have well defined regions for taxonomic classification that are not subject to horizontal transfer and have sequence databases available to researchers. Wintzingerode et al. (1997) discussed some issues surrounding differential PCR amplification including different affinities of primers to templates, different copy numbers of target genes, hybridization efficiency and primer specificity. In addition, sequences with lower G+C content are thought to separate more efficiently in the denaturing step of PCR and, therefore, could be preferentially amplified (Wintzingerode et al., 1997).

The above discusses a few limitations of molecular-based methods, which can influence the analysis and interpretation of molecular-based microbial community analysis. Molecular-based methods provide valuable information about the microbial community as opposed to only culture-based techniques.

2.4. Taxonomic ambiguity of microbes

Another problem associated with measuring microbial diversity in soil is the problem of defining microbial species (Torsvik et al., 1998; Trevors, 1998b; Ovreas, 2000). There is no official definition of a bacterial (Colwell et al., 1995) or AMF species. Moreover, Hey (2001) listed over 24 definitions of species, all of which were different. The traditional species definition was based on higher plants and animals and does not readily apply to prokaryotes (Godfray and Lawton, 2001) or asexual organisms. The genetic plasticity of bacteria, allowing DNA transfer through plasmids, bacteriophages and transposons, complicates the concept of bacterial species.

Fungal taxonomy has similar problems in identifying vegetative structures. Most of the current taxonomy is based on fungal sexual states, for instance mushrooms and truffles, and problems exist when trying to identify the below-ground vegetative structures (Horton, 2002). Molecular methods such as

restriction analysis of the internal transcribed spacer (ITS) region, 18S rDNA and restriction fragment length polymorphism (RFLP) analysis has been used to identify fungi; however, the databases are still not sufficiently developed to prevent many “unknowns” in a community analysis.

AMF taxonomy is also transient. Traditionally, AMF were identified using spore morphology and differential staining. This technique made it difficult to identify morphologically similar spores, to determine phylogenetic relatedness and to accurately classify AMF. Recently, Redecker (2000) and Redecker et al. (1997, 1999, 2000) have put considerable effort into determining the phylogenetic relationships of AMF and this led to the identification of two new families. As more research is conducted, the specificity and sensitivity of molecular techniques to identify AMF species increase and so does the ability to study these microorganisms.

Another major limitation of the use of molecular techniques with AMF is our lack of understanding of genetic polymorphism in AMF. One single spore can contain considerable variation in ITS and 5.8S rDNA sequences (Redecker et al., 1999). Therefore, the single-sequence, single-species hypothesis may not fit with AMF and diversity could be overestimated (Dodd et al., 2000; Schubler et al., 2001).

3. Methods of studying microbial diversity in soil

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species (Trevors, 1998b; Ovreas, 2000). Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques (Table 1) and molecular-based techniques (Table 2). Typically, diversity studies include the relative diversities of communities across a gradient of stress, disturbance or other biotic or abiotic difference (Hughes et al., 2001). It is difficult with current techniques to study true diversity since we do not know what is present and we have no way of determining the accuracy of our extraction or detection methods. Often researchers will attempt to reduce the information gathered by diversity studies into discrete, numerical measurements such as diversity indices (Atlas and Bartha, 1993).

4. Biochemical-based techniques to study microbial diversity

Most methods described below can be used for either bacteria or fungi, although some are specific to one or the other.

4.1. Plate counts

Traditionally, diversity was assessed using selective plating and direct viable counts. These methods are fast, inexpensive and can provide information on the active, heterotrophic component of the population. Limitations include the difficulty in dislodging bacteria or spores from soil particles or biofilms, growth medium selections (Tabacchioni et al., 2000), growth conditions (temperature, pH, light), the inability to culture a large number of bacterial and fungal species with current techniques and the potential for colony–colony inhibition or of colony spreading (Trevors, 1998b). In addition, plate growth favours those microorganisms with fast growth rates and those fungi that produce large numbers of spores (Dix and Webster, 1995). All of these limitations can influence the apparent diversity of the microbial community.

4.2. Sole carbon source utilization patterns/community level physiological profiling for measuring microbial diversity

Garland and Mills (1991) developed a technique using a commercially available 96-well microtitre plate to assess the potential functional diversity of the bacterial population through sole source carbon utilization (SSCU) patterns. Gram-negative (GN) and gram-positive (GP) plates are available from Biolog (Hayward, CA, USA, www.biolog.com) and each contains 95 different carbon sources and one control well without a substrate. GN and GP plates were developed originally for characterization of clinical bacterial isolates and not for community analysis. Subsequently, Biolog introduced an Eco-plate (Choi and Dobbs, 1999) containing 3 replicates of 31 different environmentally relevant carbon sources and one control well per replicate. Carbon sources not found in GN plates include D-cellobiose, D-xylitol, D-malic acid, L-arginine, 2-hydroxybenzoic

Table 1

Advantages and disadvantages of biochemical-based methods to study soil microbial diversity

Method	Advantages	Disadvantages	Selected references
Plate counts	Fast Inexpensive	Unculturable microorganisms not detected Bias towards fast growing individuals Bias towards fungal species that produce large quantities of spores	Tabacchioni et al. (2000), Trevors (1998b)
Community level physiological profiling (CLPP)	Fast Highly reproducible Relatively inexpensive Differentiate between microbial communities Generates large amount of data Option of using bacterial, fungal plates or site specific carbon sources (Biolog)	Only represents culturable fraction of community Favours fast growing organisms Only represents those organisms capable of utilizing available carbon sources Potential metabolic diversity, not in situ diversity Sensitive to inoculum density	Classen et al. (2003), Garland (1996a), Garland and Mills (1991)
Fatty acid methyl ester analysis (FAME)	No culturing of microorganisms, direct extraction from soil Follow specific organisms or communities	If using fungal spores, a lot of material is needed Can be influenced by external factors Possibility results can be confounded by other microorganisms	Graham et al. (1995), Siciliano and Germida (1998), Zelles (1999)

acid and 4-hydroxybenzoic acid. For a list of all 31 carbon sources, please refer to Choi and Dobbs (1999). Alternatively, researchers can use plates containing the growth medium and a tetrazolium salt from Biolog, and add site-specific carbon sources to analyze their samples (Campbell et al., 1997; Becker and Stottmeister, 1998). The tetrazolium salt changes colour as the substrate is metabolized. Since many fungal species are not capable of reducing the tetrazolium salt, Biolog developed fungal specific plates SFN2 and SFP2, which have the same substrates as GN and GP plates but without the tetrazolium salt (Classen et al., 2003). Inoculated populations are monitored over time for their ability to utilize substrates and the speed at which these substrates are utilized. Multivariate analysis is applied to the data and relative differences between soil functional diversity can be assessed.

This method has been used successfully to assess potential metabolic diversity of microbial communities in contaminated sites (Derry et al., 1998; Konopka et al., 1998), plant rhizospheres

(Ellis et al., 1995; Garland, 1996a; Grayston and Campbell, 1996; Grayston et al., 1998), arctic soils (Derry et al., 1999), soil treated with herbicides (el Fantroussi et al., 1999) or inoculation of microorganisms (Bej et al., 1991). For example, Roling et al. (2000) used the Biolog system together with DGGE to study the anaerobic microbial community in an aquifer. They found that both the anaerobic community level physiological profiling (CLPP) and denaturing gradient gel electrophoresis (DGGE) were able to separate microbial communities from the polluted aquifer below a landfill site from those of aquifers located up or downstream of the landfill. Derry et al. (1999) used GN Biolog plates to assess the functional diversity of microorganisms in three different Arctic soils incubated at different temperatures. They found significant differences in Shannon indices, substrate utilization richness and evenness at incubation temperatures that Arctic soil would be exposed to. el Fantroussi et al. (1999) used Biolog plates in conjunction with DGGE to assess the impact of three different phenylurea

Table 2

Advantages and disadvantages of some molecular-based methods to study soil microbial diversity

Method	Advantages	Disadvantages	Selected references
Guanine plus cytosine (G+C)	Not influenced by PCR biases Includes all DNA extracted Quantitative Includes rare members of community	Requires large quantities of DNA Dependent on lysing and extraction efficiency Coarse level of resolution	Nusslein and Tiedje (1999), Tiedje et al. (1999)
Nucleic acid reassociation and hybridization	Total DNA extracted Not influenced by PCR biases Study DNA or RNA Can be studied in situ	Lack of sensitivity Sequences need to be in high copy number to be detected Dependent on lysing and extraction efficiency	Torsvik et al. (1990a,b, 1996), Cho and Tiedje (2001)
DNA microarrays and DNA hybridization	Same as nucleic acid hybridization Thousands of genes can be analyzed If using genes or DNA fragments, increased specificity	Only detect most abundant species Need to be able to culture organisms Only accurate in low diversity systems	Hubert et al. (1999), Cho and Tiedje (2001), Greene and Voordouw (2003)
Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)	Large number of samples can be analyzed simultaneously Reliable, reproducible and rapid	PCR biases Dependent on lysing and extraction efficiency Sample handling can influence community, i.e. if stored too long before extraction, community can change One band can represent more than one species (co-migration) Only detects dominant species	Muyzer et al. (1993), Duineveld et al. (2001), Maarit-Niemi et al. (2001)
Single strand conformation polymorphism (SSCP)	Same as DGGE/TGGE No GC clamp No gradient	PCR biases Some ssDNA can form more than one stable conformation	Lee et al. (1996), Tiedje et al. (1999)
Amplified ribosomal DNA restriction analysis (ARDRA) or restriction fragment length polymorphism (RFLP)	Detect structural changes in microbial community	PCR biases Banding patterns often too complex	Liu et al. (1997), Tiedje et al. (1999)
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP Can be automated; large number of samples Highly reproducible Compare differences in microbial communities	Dependent on extraction and lysing efficiency PCR biases Type of Taq can increase variability Choice of universal primers Choice of restriction enzymes will influence community fingerprint	Tiedje et al. (1999), Dunbar et al. (2000), Osborn et al. (2000)
Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA)	Highly reproducible community profiles	Requires large quantities of DNA	Fisher and Triplett (1999)

Table 2 (continued)

Method	Advantages	Disadvantages	Selected references
	Can be automated (ARISA)	Resolution tends to be low-PCR biases	

herbicides on soil microbial communities. They reported that soil diversity seemed to decrease with the application of the herbicides and that principal component analysis (PCA) was able to distinguish between treated and nontreated communities. Similar in principle to the Biolog system is the API system (Merieux, France). There are a number of API strips available with various carbon sources that can be used to measure functional diversity (Torsvik et al., 1990b).

In principle, Biolog and API systems provide a community level physiological profile (CLPP) or a metabolic profile of the bacterial or fungal community's ability to utilize specific carbon sources. CLPPs can differentiate between microbial communities, are relatively easy to use, reproducible and produce a large amount of data reflecting metabolic characteristics of the communities (Zak et al., 1994). Limitations of metabolic profiling are: the methods select for only culturable microorganisms capable of growing under the experimental conditions (Garland and Mills, 1991), favours fast growing microorganisms (Yao et al., 2000), is sensitive to inoculum density (Garland, 1996b) and reflects the potential, and not the in situ, metabolic diversity (Garland and Mills, 1991). For instance, a species not active or representing only a minor fraction of the in situ population may have a competitive advantage within the Biolog well and the metabolic profile may overestimate the contribution of this species in situ. In addition, the carbon sources may not be representative of those present in soil (Yao et al., 2000) and therefore the usefulness of the information can be questioned. Nonetheless, CLPP is useful when studying the functional diversity of soils and is a valuable tool especially when used in conjunction with other methods.

4.3. Fatty acid methyl ester (FAME) analysis

A biochemical method that does not rely on culturing of microorganisms is fatty acid methyl ester

(FAME) analysis. This method provides information on the microbial community composition based on groupings of fatty acids (Ibekwe and Kennedy, 1998). Fatty acids make up a relatively constant proportion of the cell biomass and signature fatty acids exist that can differentiate major taxonomic groups within a community. Therefore, a change in the fatty acid profile would represent a change in the microbial population. It has been used to study microbial community composition and population changes due to chemical contaminants (Siciliano and Germida, 1998; Kelly et al., 1999) and agricultural practices (Bossio et al., 1998; Ibekwe and Kennedy, 1998). The readers are referred to a comprehensive review of the use of fatty acid patterns of phospholipids and lipopolysaccharides to characterize microbial populations by Zelles (1999).

For FAME analysis, fatty acids are extracted directly from soil, methylated and analyzed by gas chromatography (Ibekwe and Kennedy, 1999). FAME profiles of different soils can be compared using multivariate analysis. This method will detect changes in the composition of the bacterial and/or fungal community, as well as enable one to follow signature fatty acids of different groups of microorganisms. Ibekwe and Kennedy (1998) used phospholipid fatty acid analysis (PLFA) and CLPP to study microbial communities in the rhizosphere of plants from the field and from greenhouse pots. Principle component analysis of PLFA showed a clear distinction between field and greenhouse microbial communities, and these results corresponded to those of Biolog plates. Bossio et al. (1998) used phospholipid fatty acid profiles to detect changes in microbial communities consistent with different farming practices. But when these researchers calculated the Shannon diversity index based on PLFA relative abundance, no difference was detected. This could be because although the community was structurally different, diversity was not, or it could represent some problems with using fatty acid profiles to measure diversity (Bossio et al., 1998).

Although FAME analysis is one method to study microbial diversity, if using total organisms, fatty acid analysis is a poor method fraught with limitations. If using fungal spores to study the potential fungal diversity, approximately 130 to 150 spores are needed (Graham et al., 1995) and this may obscure detection of minor species in the population. Cellular fatty acid composition can be influenced by factors such as temperature and nutrition, and the possibility exists that other organisms can confound the FAME profiles (Graham et al., 1995). In addition, individual fatty acids cannot be used to represent specific species because individuals can have numerous fatty acids and the same fatty acids can occur in more than one species (Bossio et al., 1998).

5. Molecular-based techniques to study microbial diversity

A number of approaches have been developed to study molecular microbial diversity. These include DNA reassociation, DNA–DNA and mRNA:DNA hybridization, DNA cloning and sequencing, and other PCR-based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA).

5.1. Guanine plus cytosine (G+C) content

Differences in the guanine plus cytosine (G+C) content of DNA can be used to study the bacterial diversity of soil communities (Nusslein and Tiedje, 1999). It is based on the knowledge that microorganisms differ in their G+C content and that taxonomically related groups only differ between 3% and 5% (Tiedje et al., 1999). This method provides a coarse level of resolution as different taxonomic groups may share the same G+C range. Advantages of G+C analysis are that it is not influenced by PCR biases, it includes all DNA extracted, it is quantitative and it can uncover rare members in the microbial populations. It does, however, require large quantities of DNA (up to 50 µg) (Tiedje et al., 1999).

Nusslein and Tiedje (1999) used G+C content together with amplified ribosomal DNA restriction

analysis (ARDRA) abundance patterns and rDNA sequence analysis to study the changes in microbial diversity from a vegetative cover of forest to pasture in a Hawaiian soil. All three methods detected differences in the microbial community revealing that plants have a strong influence on the microbial community composition. Since all three methods examined the community at a different level of resolution, the authors believed that they form a complementary group of tests to more thoroughly study the microbial community.

5.2. Nucleic acid reassociation and hybridization

DNA reassociation is a measure of genetic complexity of the microbial community and has been used to estimate diversity (Torsvik et al., 1990a,b, 1996). Total DNA is extracted from environmental samples, purified, denatured and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease (Theron and Cloete, 2000). Under specific conditions, the time needed for half of the DNA to reassociate (the half association value $C_{0t1/2}$) can be used as a diversity index, as it takes into account both the amount and distribution of DNA reassociation (Torsvik et al., 1998). Alternatively, the similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics (Griffiths et al., 1999).

Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology (Schramm et al., 1996; Guo et al., 1997; Griffiths et al., 1999; Clegg et al., 2000; Theron and Cloete, 2000). These hybridization techniques can be done on extracted DNA or RNA, or in situ. Oligonucleotide or polynucleotide probes designed from known sequences ranging in specificity from domain to species can be tagged with markers at the 5'-end (Theron and Cloete, 2000). Fluorescent markers commonly used include derivatives of fluorescein or rhodamine. Quantitative dot-blot hybridization is used to measure the relative abundance of a certain group of microorganisms. The sample is lysed to release all nucleic acids. rRNA sequences of interest are quantified relative

to total rRNA by dot-blot hybridization with specific and universal oligonucleotide primers. The relative abundance may represent changes in the abundance in the population or changes in the activity and hence the amount of rRNA content (Theron and Cloete, 2000). Hybridization can also be conducted at the cellular level and can be done in situ. This provides valuable spatial distribution information on microorganisms in environmental samples. Traditionally, radioactive isotopes were used to label oligonucleotide probes, but recently fluorescent probes are often preferred. The samples are fixed to increase permeability of the cells but still maintaining cellular structure and integrity. The sample can either be attached to microscope slides or hybridized in suspension. Fluorescently labelled primers are added and allowed to hybridize, excess is washed away and the hybridized cells detected (Head et al., 1998). The method, known as fluorescent in situ hybridization or FISH has been used successfully to study the spatial distribution of bacteria in biofilms (Schramm et al., 1996).

One limitation of in situ hybridization or hybridization of nucleic acids extracted directly from environmental samples is the lack of sensitivity. Unless sequences are present in high copy number, i.e. from dominant species, they probably will not be detected. PCR eliminates this problem. DNA extracted directly from the environment can act as a template for PCR or mRNA can be reverse-transcribed into cDNA and then amplified using PCR (van Elsas and Wolters, 1995). The use of mRNA in diversity studies will allow a snapshot of the active microbial population, whereas DNA extracted directly from environmental samples can represent active as well as dormant microorganisms. The amplified PCR product can be hybridized with either oligonucleotide probes to provide specific information on the community or with other environmental samples to which microbial community similarity is compared.

5.3. DNA microarrays

More recently, DNA–DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species (Cho and Tiedje, 2001) or to assess microbial diversity (Greene and Voordouw, 2003).

This tool could be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences (Cho and Tiedje, 2001) with high specificity. The microarray can either contain specific target genes such as nitrate reductase, nitrogenase or naphthalene dioxygenase to provide functional diversity information or can contain a sample of environmental “standards” (DNA fragments with less than 70% hybridization) representing different species found in the environmental sample (Greene and Voordouw, 2003).

Reverse sample genome probing (RSGP) is a method used to analyze microbial community composition of the most dominant culturable species and uses genome microarrays. RSGP has four steps: (1) isolation of genomic DNA from pure cultures; (2) cross-hybridization testing to obtain DNA fragments with less than 70% cross-hybridization. DNA fragments with greater than 70% cross-hybridization are considered the same species. (3) Preparation of genome arrays onto a solid support; and (4) random labelling of a defined mixture of total community DNA and internal standard (Greene and Voordouw, 2003). This method has been used to analyze microbial communities in oil fields (Voordouw et al., 1991, 1992, 1993), and in contaminated soils (Shen et al., 1998; Hubert et al., 1999; Greene et al., 2000). Voordouw et al. (1993) used RSGP to study the microbial population in produced waters and on corrosion coupons of a select number of Western Canadian oil fields using 16 distinct genomes of sulfate-reducing bacteria (SRB) and 4 of heterotrophs. These authors found that SRB were more common on corrosion plug surfaces than heterotrophs and they could be contributing to the corrosion of the metal. RSGP is a useful technique when diversity is low, but several authors have had difficulty when assessing community composition of diverse habitats (Greene and Voordouw, 2003). If diversity is high, then cross-hybridization can be a problem or interpretation of the results is difficult. This was the case for Bagwell and Lovell (2000) who were studying the long-term effects of fertilization on diazotroph communities.

Like DNA–DNA hybridization, the use of RSGP and microarrays has the advantage that it is not confounded by PCR biases and microarrays can contain thousands of target gene sequences. However,

er, it only detects the most abundant species. In general, the species need to be cultured, but in principle cloned DNA fragments of unculturable could be used. The diversity has to be minimal or enriched cultures used, otherwise cross-hybridization can become problematic.

Using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of organisms growing as genes can be cloned into plasmids or PCR used to continually amplify the DNA fragments. In addition, fragments would increase the specificity of hybridization over the use of genomes and functional genes in the community could be assessed (Greene and Vourdouw, 2003).

5.4. PCR-based approaches

PCR targeting the 16S rDNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships (Pace, 1996, 1997, 1999). 18S rDNA and internal transcribed spacer (ITS) regions are increasingly used to study fungal communities. However, the available databases are not as extensive as for prokaryotes (Prosser, 2002). Initially, molecular-based methods for ecological studies relied on cloning of target genes isolated from environmental samples (Muyzer and Smalla, 1999). Although sequencing has become routine, sequencing thousands of clones is cumbersome (Tiedje et al., 1999). Therefore, many other techniques have been developed to assess microbial community diversity. In these methods, DNA is extracted from the environmental sample and purified. Target DNA (16S, 18S or ITS) is amplified using universal or specific primers and the resulting products are separated in different ways.

5.4.1. Denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two similar methods for studying microbial diversity. These techniques were originally developed to detect point mutations in DNA sequences. Muyzer et al. (1993) expanded the use of DGGE to

study microbial genetic diversity. DNA is extracted from soil samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The 5'-end of the forward primer contains a 35–40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. This is necessary so that separation on a polyacrylamide gel with a gradient of increasing concentration of denaturants (formamide and urea) will occur based on melting behaviour of the double-stranded DNA. If the GC-clamp is absent, the DNA would denature into single strands. On denaturation, DNA melts in domains, which are sequence specific and will migrate differentially through the polyacrylamide gel (Muyzer, 1999). Theoretically, DGGE can separate DNA with one base-pair difference (Miller et al., 1999). TGGE uses the same principle as DGGE except the gradient is temperature rather than chemical denaturants.

DGGE/TGGE have the advantages of being reliable, reproducible, rapid and somewhat inexpensive. Multiple samples can also be analyzed concurrently, making it possible to follow changes in microbial populations (Muyzer, 1999). Limitations of DGGE/TGGE include PCR biases (Wintzingerode et al., 1997), laborious sample handling, as this could potentially influence the microbial community, (Muyzer, 1999; Theron and Cloete, 2000), and variable DNA extraction efficiency (Theron and Cloete, 2000). It is estimated that DGGE can only detect 1–2% of the microbial population representing dominant species present in an environmental sample (MacNaughton et al., 1999). In addition, DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species (Gelsomino et al., 1999) and one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences. (Gelsomino et al., 1999; Maarit-Niemi et al., 2001).

Maarit-Niemi et al. (2001) used different combinations of DNA extraction and clean-up procedures and reported the method used does influence the banding pattern on DGGE gels. They reported that the Soil DNA Isolation Kit (MO Bio Laboratories Inc., Solana Beach, CA, USA) gave consistent, clear bands with the most extensive banding patterns. Gelsomino et al. (1999) found that direct and indirect

DNA extraction methods yielded DNA fingerprints that were 90% identical, with sample variation for each extraction method being less than 5%. Most of the differences in extraction methods and in reproducibility were between faint bands, presumably representing less dominant species (Gelsomino et al., 1999). Holben et al. (2004) used DGGE in combination with G+C fractionation to assess microbial community diversity and to detect minority populations of bacteria in the digestive tracts of chickens. This approach shows promise in that the fractionation reduces the complexity of the community and allows the detection of species that are present in low abundance. DGGE/TGGE has been used to assess the diversity of bacteria and fungi in the rhizosphere (Duineveld et al., 1998, 2001; Smalla et al., 2001), caused by changes of nutrient addition (Iwamoto et al., 2000) and addition of anthropogenic chemicals (Torsvik et al., 1998; el Fantroussi et al., 1999; MacNaughton et al., 1999; Whiteley and Bailey, 2000).

The partial community level fingerprints derived from DGGE/TGGE banding patterns have been analyzed for diversity studies based on the number and intensity of the DNA bands as well as similarity between treatments. However, with the limitations of PCR and of banding pattern separation, care must be exercised when interpreting results with respect to microbial diversity. Specific DGGE/TGGE bands can also be excised from gels, re-amplified and sequenced or transferred to membranes and hybridized with specific primers to provide more structural or functional diversity information (Theron and Cloete, 2000). By sequencing bands, one can obtain information about the specific microorganisms in the community. Instead of simply noting a change in the community structure based on banding patterns, one can obtain information about specific taxonomic groups within the community. While the rRNA genes have been the main target of microbial diversity studies using DGGE, some researchers have targeted catabolic genes, such as methane monooxygenase (Fjeilbirkeland et al., 2001; Knief et al., 2003) for DGGE analysis. This would provide information on the diversity of specific groups of microorganisms competent in a defined function such as pollutant degradation.

5.4.2. Single strand conformation polymorphism (SSCP)

Another technique that relies on electrophoretic separation based on differences in DNA sequences is single strand conformation polymorphism (SSCP). Like DGGE/TGGE, this technique was originally developed to detect known or novel polymorphisms or point mutations in DNA (Orita et al., 1989). Single-stranded DNA is separated on a polyacrylamide gel based on differences in mobility caused by their folded secondary structure (Lee et al., 1996). When DNA fragments are of equal size and no denaturant is present, folding and hence mobility, will be dependent on the DNA sequences. SSCP has all the same limitations of DGGE. Also, some single-stranded DNA can form more than one stable conformation. Therefore, one sequence may be represented by more than one band on the gel (Tiedje et al., 1999). However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity (Peters et al., 2000; Stach et al., 2001). SSCP has been used to measure succession of bacterial communities (Peters et al., 2000), rhizosphere communities (Schwieger and Tebbe, 1998; Schmalenberger et al., 2001), bacterial population changes in an anaerobic bioreactor (Zumstein et al., 2000) and AMF species in roots (Simon et al., 1993; Kjoller and Rosendahl, 2000).

5.4.3. Restriction fragment length polymorphism (RFLP)/amplified ribosomal DNA restriction analysis (ARDRA)

Restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA) is another tool used to study microbial diversity that relies on DNA polymorphisms. In the study by Liu et al. (1997), PCR-amplified rDNA is digested with a 4-base pair cutting restriction enzyme. Different fragment lengths are detected using agarose or non-denaturing polyacrylamide gel electrophoresis in the case of community analysis (Liu et al., 1997; Tiedje et al., 1999). RFLP banding patterns can be used to screen clones (Pace, 1996) or used to measure bacterial community structure (Massol-Deya et al., 1995). This method is useful for detecting structural changes in microbial communities but not as a measure of diversity or detection of

specific phylogenetic groups (Liu et al., 1997). Banding patterns in diverse communities become too complex to analyze using RFLP since a single species could have four to six restriction fragments (Tiedje et al., 1999). Perhaps by using a six-base cutting enzyme, the number of restriction fragments per species could be reduced, thereby increasing the resolution of this method.

5.4.4. Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of the limitations of RFLP (Tiedje et al., 1999). It follows the same principle as RFLP except that one PCR primer is labelled with a fluorescent dye, such as TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). This allows detection of only the labelled terminal restriction fragment (Liu et al., 1997). This simplifies the banding pattern, thus allowing the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Tiedje et al., 1999). The banding pattern can be used to measure species richness and evenness as well as similarities between samples (Liu et al., 1997). This procedure can be automated to allow sampling and analysis of a large number of soil samples (Osborn et al., 2000). Osborn et al. (2000) tested the reproducibility of the method and found that banding patterns within and between samples were highly reproducible. They did find that the use of different Taq polymerases increased variability of the same DNA sample. T-RFLP is limited not only by DNA extraction and PCR biases, but also by the choice of universal primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial and archaeal domains. Additionally, these primers are based on existing 16S rRNA, 18S rRNA or ITS databases, which until recently contained mainly sequences from culturable microorganisms, and therefore may not be representative of the true microbial diversity in a sample (Liu et al., 1997). In addition, different enzymes will produce different community fingerprints (Dunbar et al., 2000). It is therefore, important to use at least two to four

different restriction enzymes (Tiedje et al., 1999). T-RFLP, like any PCR-based method, may underestimate true diversity because only numerically dominant species are detected because of the large quantity of available template DNA. In addition, different species will have different gene copy numbers and this could bias results (Liu et al., 1997). Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn et al., 2000). Despite these limitations, some researchers are of the opinion that once standardized, T-RFLP can be a useful tool to study microbial diversity in the environment (Liu et al., 1997; Tiedje et al., 1999; Osborn et al., 2000), while others feel that it is inadequate (Dunbar et al., 2000). Dunbar et al. (2000) reported that the statistics they used detected inconsistencies in DNA banding patterns depending on the enzyme used and that samples of four different soil types were not found to be significantly different from each other. T-RFLP has also been thought to be an excellent tool with which to compare the relationship between different samples (Dunbar et al., 2000).

T-RFLP has been used to measure spatial and temporal changes in bacterial communities (Acinas et al., 1997; Lukow et al., 2000), to study complex bacterial communities (Clement et al., 1998; Moeseneder et al., 1999), to detect and monitor populations (Tiedje et al., 1999) and to assess the diversity of AMF in the rhizosphere of *Viola calaminaria* in a metal-contaminated soil (Tonin et al., 2001). Tiedje et al. (1999) reported five times greater success at detecting and tracking specific ribotypes using T-RFLP than DGGE.

5.4.5. Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA)

Similar in principle to RFLP and T-RFLP, RISA and ARISA provide ribosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence

(Fisher and Triplett, 1999). In RISA, the sequence polymorphisms are detected using silver stain while in ARISA the forward primer is fluorescently labelled and is automatically detected (Fisher and Triplett, 1999). Both methods provide highly reproducible bacterial community profiles but RISA requires large quantities of DNA, is more time-consuming, silver staining is somewhat insensitive and resolution tends to be low (Fisher and Triplett, 1999). ARISA increases the sensitivity of the method and reduces the time but is still subject to the traditional limitations of PCR (Fisher and Triplett, 1999). RISA has been used to compare microbial diversity in soil (Borneman and Triplett, 1997), in the rhizosphere of plants (Borneman and Triplett, 1997), in contaminated soil (Ranjard et al., 2000) and in response to inoculation (Yu and Mohn, 2001).

5.4.6. Highly repeated sequence characterization or microsatellite regions

Many organisms, both prokaryotic and eukaryotic, contain highly repetitive short DNA sequences that are 1–10 base pairs long repeated throughout their genomes (Zeze et al., 1996; Longato and Bonfante, 1997; Tiedje et al., 1999). Depending on the rate of evolution, these sequences may be diagnostic and allow differentiation down to the species or strain level (Zeze et al., 1996). This method, also termed *rep*-PCR, has been used for identification of bacteria since it provides a genomic fingerprint of chromosome structure, and chromosome structure is considered to be variable between strains (Tiedje et al., 1999). Highly repeated sequences are also referred to as microsatellite regions and have been used for identification of mycorrhizal fungi (Longato and Bonfante, 1997). Fingerprinting of PCR-amplified microsatellites can be compared using similarity indices to investigate difference at the inter- and intraspecific level (Longato and Bonfante, 1997). The use of this method to study microbial diversity may be limited depending on the complexity of the community; however, it may be useful to develop probes to detect changes in the microbial community caused by an environmental change. Another limitation of this method is that the sequence of the microsatellite region needs to be known so appropriate primers can be used.

6. Selected results of soil microbial diversity

There is extensive research on soil microbial diversity. Here we will describe a few selected studies which have used more than one method to study microbial diversity. Ellis et al. (2003) compared the effects of metal contamination on culturable and non-culturable diversity of soil microorganisms in five different soils using plate counts and DGGE. Their study was aimed to test the hypothesis that the readily culturable soil bacteria may be the main contributor to ecosystem functioning. They found that direct amplification of rRNA genes from environmental samples gave similar DGGE banding patterns for all treatments but that DGGE patterns from plate washes of culturable bacteria differed significantly from each other and from their total community pattern. Also, the culturability of microorganisms was greatly reduced in samples with the highest metal contamination. These authors concluded that the culturable portion of the microbial community is an important ecological parameter and it is important to assess activity as opposed to the presence or absence of bacteria.

Muller et al. (2001) used a variety of biochemical and molecular techniques to assess the impact of mercury pollution on the soil microbial community from three different sites with varying levels of contamination. Biomass, colony morphology typing, ARDRA and DGGE all detected differences in the soil with the highest level of contamination (511 total mercury $\mu\text{g g dw soil}^{-1}$) compared to the lowest and intermediate level of contamination (7 and 28 total mercury $\mu\text{g g dw soil}^{-1}$, respectively). Sole carbon source utilization pattern analysis did not detect any differences between the number of substrates utilized or the similarity of their utilization but the soil with the highest level of contamination did separate from the other two soils in PCA analysis. The authors were unclear if the mercury had influencing the assay.

In a follow-up study, Muller et al. (2002) used colony morphology typing, DGGE profiling and substrate utilization patterns to study how two different disturbances, mercury contamination and tylosin (a veterinary antibiotic) treatment affect the diversity and function of soil microbial communities. Microbial function was assessed using respiration after the

addition of a substrate (glucose or alfalfa) following yet another disturbance, a heat treatment. These researchers detected differences in diversity using both morphology typing and DGGE, especially for mercury contamination, but no differences were detected using substrate utilization data. The authors felt that the substrate utilization data examined a different level of the microbial community and was attributed to a consortium of bacteria capable of growth in the individual wells as opposed to individual species. The authors did notice an increased lag time and respiration delay in mercury-contaminated treatments especially upon the addition of a complex substrate such as alfalfa. It is noted that the treatments with decreased diversity in both culturable and non-culturable microbial fractions also exhibited increased lag phase and respiration delay after the addition of another stress. The results from this study could support the hypothesis that reduced diversity leads to reduced ecosystem stability and hence ecosystem functioning.

Siciliano et al. (2003) used DGGE, as well as DNA hybridization with catabolic gene probes (*ndoB*, *alkB* and *xylE*) of culturable bacterial colonies, to assess the structural and functional diversity of bulk soil microbial communities in soil contaminated with polycyclic aromatic hydrocarbons (PAHs) during a phytoremediation trial. They hypothesized that the plant's influence on the soil microbial community would extend beyond the rhizosphere and would alter the functioning of the bulk soil microbial community to aid in degradation. They found that tall fescue (*Festuca arundinacea*) altered the functional catabolic diversity by increasing the prevalence of catabolic genes in the bulk soil, but that this enrichment was not detected using DGGE.

Ibekwe et al. (2002) used PLFA and DGGE together to assess the impacts of agricultural management techniques, such as no-tillage and conventional tillage, and environmental influences, such as precipitation, of four different soils. They found that the information obtained from both techniques complemented each other as they had a common pattern of clustering for the four soils. The no-till and the conservation reserve program clustered closer together than the conventional till and minimal tillage in the PCA analysis of PLFA, and the similarity and correspondence analysis of DGGE patterns. The

authors concluded that management techniques (tillage) impacted the microbial community composition more than the amount of precipitation.

The authors of the above studies used various techniques to determine the effects of pollutants, farming practices and precipitation on the microbial community structure and/or function. It can be concluded that the best method(s) to use depend on the questions being asked and the resources available. DGGE, ARDRA, PLFA and colony morphologies all detected changes in the microbial population structure and diversity (Ibekwe et al., 2002; Muller et al., 2001, 2002). When the results of DGGE and PLFA were compared (Ibekwe et al., 2002), the results complemented each other. However, when substrate utilization patterns were used with these other techniques, it did not detect any corresponding changes in the potential metabolic functional diversity of the microbial communities, despite the changes in structure detected.

The degree of resolution of carbon source utilization patterns seems to be at a community functional level since it is performed by a consortium of bacteria. Since functional redundancy is thought to exist in soil bacteria, it is expected that carbon source utilization patterns would only detect a drastic change in the microbial community. Where DGGE, ARDRA, PLFA and colony morphologies are all detecting changes in community structure potentially to a species level, and therefore are expected to detect smaller shifts in the community. DGGE and ARDRA are both molecular based and PLFA is a direct extraction of phospholipid fatty acids from soil. Therefore, all three methods are examining both the culturable and non-culturable fractions of the soil microbial community where colony morphology depends only on the culturable fraction.

There is a wide range of methods available to study soil microbial diversity. Each method has its limitations and only provides a partial picture of one aspect of soil microbial diversity. Since it is impossible to evaluate the effectiveness of each method with our current knowledge, it is advised that researchers study the microbial population on as many different levels as possible. As the proceeding examples illustrate, a broader, more complete picture of soil microbial diversity can be obtained by using a variety of methods, each with a different endpoint, to provide a more global assessment of changes in

microbial structure and function. One should be aware that changes in microbial community diversity in a habitat may not imply deleterious effects. Thus the need to learn how changes in microbial community structure influence microbial community function is apparent.

7. Future perspectives

It is important to study microbial diversity not only for basic scientific research, but also to understand the link between diversity and community structure and function. Human influences such as pollution, agriculture and chemical applications could adversely affect microbial diversity, and perhaps also above- and below-ground ecosystem functioning. For instance, Buckley and Schmidt (2001) found significantly higher amounts of 16S rRNA for all microbial groups analyzed in fields that have never been cultivated as compared to agricultural fields. This suggests a decrease in bacterial biomass or activity in cultivated fields. Similarly, the diversity of AMF has been shown to increase from arable fields to natural systems (Daniell et al., 2001; Menendez et al., 2001). However, it is not known what these reductions in diversity mean to ecosystem functioning and it is important for sustainability of ecosystems that the link between diversity and function be examined and better understood.

There is disagreement within the scientific community of whether taxonomic or genetic diversity is important as long as functional diversity is maintained. Given the limitations of our ability to study diversity and how diversity relates to function, it would be prudent to assume functional redundancy does not exist and taxonomic diversity is important to maintain. It was once thought that AMF were functionally redundant given a lack of host specificity, but it has since been found that they are not functionally redundant and do provide different benefits to different plant hosts.

Knowledge of microbial diversity and function in soils is limited because of the taxonomic and methodological limitations associated with studying these organisms. Although methods to study diversity (numerical, taxonomic, structural) are improving for both bacteria and fungi, there is still not a clear

association between diversity and function. Even if an organism is functionally redundant in one function, chances are it is not redundant in all functions and will have different susceptibilities and tolerances to abiotic and biotic stresses. It is generally thought that a diverse population of organisms will be more resilient to stress and more capable of adapting with environmental changes.

Bacterial and fungal diversity increases soil quality by affecting soil agglomeration and increasing soil fertility. They are both important in nutrient cycling and in enhancing plant health through direct or indirect means. In addition, a healthy rhizosphere population can help plants deal with biotic and abiotic stresses such as pathogens, drought and soil contamination.

Our current ability to study and understand soil microbial diversity is wrought with taxonomic and methodological limitations. Soil microbiologists face the difficult task of attempting to define and identify microorganisms and their functions. This paper summarizes some of the common biochemical and molecular methods used to study soil microbial communities. Although molecular methods have the advantage of obtaining information about non-culturable organisms, they also have limitations that cannot be ignored. It is challenging to soil microbiologists to develop techniques to study soil microbial diversity when it is currently impossible to know how accurate these techniques are. We do not know what is present in a gram of soil, and therefore it is difficult to conclude whether one technique of studying diversity is better than another. Given the current state of knowledge, we feel that the best way to study soil microbial diversity would be to use a variety of tests with different endpoints and degrees of resolution to obtain the broadest picture possible and the most information regarding the microbial community. In addition, methods to understand the link between structural diversity and functioning of below- and above-ground ecosystems need to be developed so that the question of how diversity influences function can be addressed.

Our knowledge of plant–microbe–soil interactions is increasing, but the complexity of interacting biological, chemical and physical factors means that much remains to be understood. As new techniques are developed, our level of understanding increases and our knowledge expands.

Acknowledgements

We would like to thank Premier Tech (Riviere du Loup, Quebec, Canada) for partial funding and support. J.T.T., H.L. and J.N.K. sincerely acknowledge financial support from the Natural Sciences and Engineering Research Council (NSERC) of Canada. J.L.K. was the recipient of an Industrial NSERC award and an Ontario Graduate Scholarship. We also thank the Canadian Foundation for Innovation and Ontario Challenge Fund for infrastructure and equipment support.

References

- Acinas, S., Rodriguez-Valera, R., Pedros-Alio, C., 1997. Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16S rDNA. *FEMS Microbiol. Ecol.* 24, 27–40.
- Atlas, R.M., Bartha, R., 1993. *Microbial Ecology Fundamentals and Applications*. 3rd ed. Benjamin Cummings Publishing, New York.
- Bagwell, C.E., Lovell, C.R., 2000. Persistence of selected *Spartina alterniflora* rhizoplane diazotrophs exposed to natural and manipulated environmental variability. *Appl. Environ. Microbiol.* 66, 4625–4633.
- Becker, P.M., Stottmeister, U., 1998. General (Biolog GN) versus site-relevant (pollutant-dependent) sole-carbon-source utilization patterns as a means to approaching community functioning. *Can. J. Microbiol.* 44, 913–919.
- Bej, A.K., Perlin, M., Atlas, R.M., 1991. Effect of introducing genetically engineered microorganisms on soil microbial community diversity. *FEMS Microbiol. Ecol.* 86, 169–176.
- Borneman, J., Triplett, E., 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial shifts associated with deforestation. *Appl. Environ. Microbiol.* 63, 2647–2653.
- Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., Nienhuis, J., Triplett, E.W., 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* 62, 1935–1943.
- Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microb. Ecol.* 36, 1–12.
- Buckley, D.H., Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. *Microb. Ecol.* 42, 11–21.
- Cairney, J.W.G., 2000. Evolution of mycorrhiza systems. *Naturwissenschaften* 87, 467–475.
- Campbell, C.D., Grayston, S.J., Hirst, D.J., 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *J. Microbiol. Methods* 30, 33–41.
- Cho, J.-C., Tiedje, J.M., 2001. Bacterial species determination from DNA–DNA hybridization by using genome fragments and DNA microarrays. *Appl. Environ. Microbiol.* 67, 3677–3682.
- Choi, K.-H., Dobbs, F.C., 1999. Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. *J. Microbiol. Methods* 36, 203–213.
- Classen, A.T., Boyle, S.I., Haskins, K.E., Overby, S.T., Hart, S.C., 2003. Community-level physiological profiles of bacteria and fungi: plate type and incubation temperature influences on contrasting soils. *FEMS Microbiol. Ecol.* 44, 319–328.
- Clegg, C.D., Ritz, K., Griffiths, B.S., 2000. %G+C profiling and cross hybridisation of microbial DNA reveals great variation in below-ground community structure in UK upland grasslands. *Appl. Soil Ecol.* 14, 125–134.
- Clement, B.G., Kehl, L.E., DeBord, K.L., Kitts, C.L., 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for comparison of complex bacterial communities. *J. Microbiol. Methods* 31, 135–142.
- Colwell, R.R., Clayton, R.A., Ortiz-Conde, B.A., Jacobs, D., Russek-Cohen, E., 1995. The microbial species concept and biodiversity. In: Allsopp, D., Colwell, R.R., Hawksworth, D.L. (Eds.), *Microbial Diversity and Ecosystem Function: Proceedings of the IUBS/IUMS Workshop held at Egham, UK, 10–13 August 1993 in support of the IUBS/UNESCO/SCOPE 'DIVERSITAS' programme*. CAB International, Cambridge, pp. 3–15.
- Curl, E.A., Truelove, B., 1986. *The Rhizosphere*. Springer-Verlag, Berlin.
- Daniell, T.J., Husband, R., Fitter, A.H., Young, J.P.W., 2001. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiol. Ecol.* 36, 203–209.
- Derry, A.M., Staddon, W.J., Trevors, J.T., 1998. Functional diversity and community structure of microorganisms in uncontaminated and creosote-contaminated soils as determined by sole-carbon-source-utilization. *World J. Microbiol. Biotechnol.* 14, 571–578.
- Derry, A.M., Staddon, W.J., Kevan, P.G., Trevors, J.T., 1999. Functional diversity and community structure of micro-organisms in three arctic soils as determined by sole-carbon-source-utilization. *Biodivers. Conserv.* 8, 205–221.
- Dix, N.J., Webster, J., 1995. *Fungal Ecology*. Chapman & Hall, London.
- Dodd, J.C., Boddington, C.L., Rodriguez, A., Gonzalez-Chavez, C., Mansur, I., 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant Soil* 226, 131–151.
- Duineveld, B.M., Rosado, A.S., van Elsas, J.D., van Veen, J.A., 1998. Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. *Appl. Environ. Microbiol.* 64, 4950–4957.
- Duineveld, B.M., Kowalchuk, G.A., Keijzer, A., van Elsas, J.D., 2001. Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 67, 172–178.
- Dunbar, J., Ticknor, L.O., Kuske, C.R., 2000. Assessment of mi-

- crobal diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl. Environ. Microbiol.* 66, 2943–2950.
- el Fantroussi, S., Verschuere, L., Verstraete, W., Top, E.M., 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol.* 65, 982–988.
- Ellis, R.J., Thompson, I.P., Bailey, M.J., 1995. Metabolic profiling as a means of characterizing plant-associated microbial communities. *FEMS Microbiol. Ecol.* 16, 9–18.
- Ellis, R.J., Morgan, P., Weightman, A.J., Fry, J.C., 2003. Cultivation-dependent and-independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl. Environ. Microbiol.* 69, 3223–3230.
- Filion, M., St-Arnaud, M., Fortin, J.A., 1999. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. *New Phytol.* 141, 525–533.
- Fisher, M.M., Triplett, E.W., 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 65, 4630–4636.
- Fjeilbirkeland, A., Torsvik, V., Ovreas, L., 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmuA*, *mxrA* and 16S rDNA sequences.
- Franklin, R.B., Mills, A.L., 2003. Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. *FEMS Microb. Ecol.* 44, 335–346.
- Garland, J.L., 1996. Patterns of potential C source utilization by rhizosphere communities. *Soil Biol. Biochem.* 28, 223–230.
- Garland, J.L., 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil Biol. Biochem.* 28, 213–221.
- Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source utilization. *Appl. Environ. Microbiol.* 57, 2351–2359.
- Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G., van Elsas, J.D., 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *J. Microbiol. Methods* 38, 1–15.
- George, E., Marschner, H., Jakobsen, I., 1995. Role of arbuscular mycorrhizal fungi in uptake of phosphorous and nitrogen from soil. *Crit. Rev. Biotechnol.* 15, 257–270.
- Giller, K.E., Beare, M.H., Lavelle, P., Izac, A.-M.N., Swift, M.J., 1997. Agricultural intensification, soil biodiversity and agroecosystem function. *Appl. Soil Ecol.* 6, 3–16.
- Godfray, H.C.J., Lawton, J.H., 2001. Scale and species numbers. *TREE* 16, 400–404.
- Graham, J.H., Hodge, N.C., Morton, J.B., 1995. Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae. *Appl. Environ. Microbiol.* 61, 58–64.
- Grayston, S.J., Campbell, C.D., 1996. Functional biodiversity of microbial communities in the rhizospheres of hybrid larch (*Larix eurolepis*) and sitka spruce (*Picea sitchensis*). *Tree Physiol.* 16, 1031–1038.
- Grayston, S.J., Wang, S., Campbell, C.D., Edwards, A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* 30, 369–378.
- Greene, E.A., Voordouw, G., 2003. Analysis of environmental microbial communities by reverse sample genome probing. *J. Microbiol. Methods* 53, 211–219.
- Greene, E.A., Kay, J.G., Jaber, K., Stehmeier, L.G., Voordouw, G., 2000. Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. *Appl. Environ. Microbiol.* 66, 5282–5289.
- Griffiths, B.S., Ritz, K., Ebbelwhite, N., Dobson, G., 1999. Soil microbial community structure: effects of substrate loading rates. *Soil Biol. Biochem.* 31, 145–153.
- Grundmann, L.G., Gourbiere, F., 1999. A micro-sampling approach to improve the inventory of bacterial diversity in soil. *Appl. Soil Ecol.* 13, 123–126.
- Guo, C., Sun, W., Harsh, J.B., Ogram, A., 1997. Hybridization analysis of microbial DNA from fuel oil-contaminated and non-contaminated soil. *Microb. Ecol.* 34, 178–187.
- Head, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35, 1–21.
- Hey, J., 2001. The mind of the species problem. *TREE* 16, 326–329.
- Horton, T.R., 2002. Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. *Plant Soil* 244, 29–39.
- Hubert, C., Shen, Y., Voordouw, G., 1999. Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. *Appl. Environ. Microbiol.* 65, 3064–3070.
- Hughes, J.B., Hellmann, J.J., Ricketts, T.H., Bohannan, B.J.M., 2001. Minireview: counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67, 4399–4406.
- Ibekwe, A.M., Kennedy, A.C., 1998. Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. *FEMS Microbiol. Ecol.* 26, 151–163.
- Ibekwe, A.M., Kennedy, A.C., 1999. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. *Plant Soil* 206, 151–161.
- Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.H., Crowley, D.E., 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiol. Ecol.* 39, 183–191.
- Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, N., Eguchi, M., Nasu, M., 2000. Monitoring impact of in situ bio-stimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.* 32, 129–141.
- Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *New Phytol.* 135, 575–585.
- Kelly, J.J., Hagglblom, M., Tate III, R.L., 1999. Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study. *Soil Biol. Biochem.* 31, 1455–1465.

- Kjoller, R., Rosendahl, S., 2000. Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). *Plant Soil* 226, 189–196.
- Klironomos, J.N., Rillig, M.C., Allen, M.F., 1999. Designing belowground field experiments with the help of semi-variance and power analyses. *Appl. Soil Ecol.* 12, 227–238.
- Klironomos, J.N., McCune, J., Hart, M., Neville, J., 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. *Ecol. Lett.* 3, 137–141.
- Knief, C., Lipski, A., Dunfield, P.F., 2003. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* 69 (11), 6703–6714.
- Konopka, A., Oliver Jr., L., Turco, R.F., 1998. The use of carbon source utilization patterns in environmental and ecological microbiology. *Microb. Ecol.* 35, 103–115.
- Lee, D.-H., Zo, Y.-G., Kim, S.-J., 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single strand conformation polymorphism. *Appl. Environ. Microbiol.* 62, 3112–3120.
- Liu, W.-t., Marsh, T.L., Cheng, H., Forney, L.J., 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 63, 4516–4522.
- Longato, S., Bonfante, P., 1997. Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycol. Res.* 101, 425–432.
- Lukow, T., Dunfield, P.F., Liesack, W., 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiol. Ecol.* 32, 241–247.
- Maarit-Niemi, R., Heiskanen, I., Wallenius, K., Lindstrom, K., 2001. Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *J. Microbiol. Methods* 45, 155–165.
- MacNaughton, S.J., Stephen, J.R., Venosa, A.D., Davis, G.A., Chang, Y.-J., White, D.C., 1999. Microbial population changes during bioremediation of an experimental oil spill. *Appl. Environ. Microbiol.* 65, 3566–3574.
- Massol-Deya, A.A., Odelson, D.A., Hickey, R.F., Tiedje, J.M., 1995. Bacterial community fingerprinting of amplified 16S and 16S–23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: Akkermans, A.D.L., van-Elsas, J.D., de-Brujin, F.J. (Eds.), *Molecular Microbial Ecology Manual*. Kluwer Academic Publishing, Boston, pp. 3.3.2.1–3.3.2.8.
- Menendez, A.B., Scervino, J.M., Godeas, A.M., 2001. Arbuscular mycorrhizal populations associated with natural and cultivated vegetation on a site of Buenos Aires province, Argentina. *Biol. Fertil. Soils* 33 (5), 373–381.
- Miller, K.M., Ming, T.J., Schulze, A.D., Withler, R.E., 1999. Denaturing Gradient Gel Electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. *BioTechniques* 27, 1016–1030.
- Moeseneder, M.M., Arrieta, J.M., Muyzer, G., Winter, C., Herndl, G.J., 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 65, 3518–3525.
- Molin, J., Molin, S., 1997. CASE: complex adaptive systems ecology. In: Jones, J.G. (Ed.), *Advances in Microbial Ecology*, vol. 15. Plenum, New York, pp. 27–79.
- Muller, A.K., Westergaard, K., Christensen, S., Sorensen, S.J., 2001. The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiol. Ecol.* 36, 11–19.
- Muller, A.K., Westergaard, K., Christensen, S., Sorensen, S.J., 2002. The diversity and function of soil microbial communities exposed to different disturbances. *Microb. Ecol.* 44, 49–58.
- Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* 2, 317–322.
- Muyzer, G., Smalla, K., 1999. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis in microbial ecology. *Antonie van Leeuwenhoek* 73, 127–141.
- Muyzer, G., Waal, E.C.D., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Nusslein, K., Tiedje, J.M., 1999. Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. *Appl. Environ. Microbiol.* 65, 3622–3626.
- O'Donnell, A.G., Seasman, M., Macrae, A., Waite, I., Davies, J.T., 2001. Plants and fertilisers as drivers of change in microbial community structure and function in soils. *Plant Soil* 232, 135–145.
- Orita, M., Suzuki, Y., Sekiya, T., Hayashi, K., 1989. A rapid and sensitive detection of point mutations and genetic polymorphisms using polymerase chain reaction. *Genomics* 5, 874–879.
- Osborn, A.M., Moore, E.R.B., Timmis, K.N., 2000. An evaluation of terminal-restriction fragment length polymorphisms (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.* 2, 39–50.
- Ovreas, L., 2000. Population and community level approaches for analysing microbial diversity in natural environments. *Ecol. Lett.* 3, 236–251.
- Ovreas, L., Jensen, S., Daae, F.L., Torsvik, V., 1998. Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. *Appl. Environ. Microbiol.* 64, 2739–2742.
- Pace, N.R., 1996. New perspective on the natural microbial world: molecular microbial ecology. *ASM News* 62, 463–470.
- Pace, N.R., 1997. A molecular view of microbial diversity and the biosphere. *Science* 276, 734–740.
- Pace, N.R., 1999. Microbial ecology and diversity. *ASM News* 65, 328–333.
- Peters, S., Koschinsky, S., Schwieger, F., Tebbe, C.C., 2000. Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation-polymorphism-based genetic profiles of small-subunit rRNA genes. *Appl. Environ. Microbiol.* 66, 930–936.
- Prosser, J.I., 2002. Molecular and functional diversity in soil microorganisms. *Plant Soil* 244, 9–17.
- Ranjard, L., Brothier, E., Nazaret, S., 2000. Sequencing bands of

- ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl. Environ. Microbiol.* 66, 5334–5339.
- Redecker, D., 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. *Mycorrhiza* 10, 73–80.
- Redecker, D., Thierfelder, H., Walker, C., Werner, D., 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Appl. Environ. Microbiol.* 63, 1756–1761.
- Redecker, D., Hijiri, M., Dulieu, H., Sanders, I.R., 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. *Fungal Genet. Biol.* 28, 238–244.
- Redecker, D., Morton, J.B., Bruns, T.D., 2000. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Mol. Phylogenet. Evol.* 14, 276–284.
- Roling, W.F.M., van-Breukelen, B.M., Braster, M., Goeltom, M.T., Groen, J., 2000. Analysis of microbial communities in a landfill leachate polluted aquifer using a new method for anaerobic physiological profiling and 16S rDNA based fingerprinting. *Microb. Ecol.* 40, 177–188.
- Rondon, M.R., Goodman, R.M., Handelsman, J., 1999. The earth's bounty: assessing and accessing soil microbial diversity. *Trends Biotech.* 17, 403–409.
- Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K.A., Lynch, B.A., MacNeil, I.A., Minor, C., Tiong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., Goodman, R.M., 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* 66, 2541–2547.
- Sanders, I.R., Fitter, A.H., 1992. The ecology and functioning of vesicular-arbuscular mycorrhizas in co-existing grassland species: I Seasonal patterns of mycorrhizal occurrence and morphology. *New Phytol.* 120, 517–524.
- Schmalenberger, A., Schwieger, F., Tebbe, C.C., 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. *Appl. Environ. Microbiol.* 67, 3557–3563.
- Schramm, A., Larsen, L.H., Revsbech, N.P., Ramsing, N.B., Amann, R., Schleifer, K.-H., 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* 62, 4641.
- Schubler, A., Gehrig, H., Schwarzott, D., Walker, C., 2001. Analysis of partial Glomales SSU rRNA gene sequences: implications for primer design and phylogeny. *Mycol. Res.* 105, 5–15.
- Schwieger, F., Tebbe, C., 1998. A new approach to utilize PCR-single-strand-conformation-polymorphism for 16S rRNA gene-based microbial community analysis. *Appl. Environ. Microbiol.* 64, 4870–4876.
- Shen, Y., Stehmeier, L.G., Voordouw, G., 1998. Identification of hydrocarbon-degrading bacteria in soil by reverse sample genome probing. *Appl. Environ. Microbiol.* 64, 637–645.
- Siciliano, S.D., Germida, J.J., 1998. Biolog analysis and fatty acid methyl ester profiles indicate that *Pseudomonas* inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*. *Soil Biol. Biochem.* 30, 1717–1723.
- Siciliano, S.D., Germida, J.J., Banks, K., Greer, C.W., 2003. Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. *Appl. Environ. Microbiol.* 69, 483–489.
- Simon, L., Levesque, R.C., Lalonde, M., 1993. Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. *Appl. Environ. Microbiol.* 59, 4211–4215.
- Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, R., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* 67, 4742–4751.
- Smith, K.P., Goodman, R.M., 1999. Host variation for interactions with beneficial plant-associated microbes. *Annu. Rev. Phytopathol.* 37, 473–491.
- Srivastava, D., Kapoor, R., Srivastava, S.K., Mukerji, K.G., 1996. Vesicular arbuscular mycorrhiza—an overview. In: Mukerji, K.G. (Ed.), *Concepts in Mycorrhizal Research*. Kluwer Academic Publishing, Netherlands, pp. 1–39.
- Stach, J.E.M., Bathe, S., Clapp, J.P., Burns, R.G., 2001. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiol. Ecol.* 36, 139–151.
- Tabacchioni, S., Chiarini, L., Bevivino, A., Cantale, C., Dalmastri, C., 2000. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. *Microb. Ecol.* 40, 169–176.
- Theron, J., Cloete, T.E., 2000. Molecular techniques for determining microbial diversity and community structure in natural environments. *Crit. Rev. Microbiol.* 26, 37–57.
- Thorn, G., 1997. The fungi in soil. In: van Elsas, J.D., Trevors, J.T., Wellington, E.M.H. (Eds.), *Modern Soil Microbiology*. Marcel Dekker, New York, pp. 63–127.
- Tiedje, J.M., Asuming-Brempong, S., Nusslein, K., Marsh, T.L., Flynn, S.J., 1999. Opening the black box of soil microbial diversity. *Appl. Soil Ecol.* 13, 109–122.
- Timonen, S., Finlay, R.D., Olsson, S., Soderstrom, B., 1996. Dynamics of phosphorous translocation in intact ectomycorrhizal systems: non-destructive monitoring using a B-scanner. *FEMS Microbiol. Ecol.* 19, 171–180.
- Tonin, C., Vandenkoornhuyse, P., Joner, E.J., Straczek, J., Leyval, C., 2001. Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover. *Mycorrhiza* 10, 161–168.
- Torsvik, V., Goksoyr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* 56, 782–787.
- Torsvik, V., Salte, K., Soerheim, R., Goksoyr, J., 1990. Comparison of phenotypic diversity and DNA heterogeneity in a

- population of soil bacteria. *Appl. Environ. Microbiol.* 56, 776–781.
- Torsvik, V., Sorheim, R., Goksoyr, J., 1996. Total bacterial diversity in soil and sediment communities—a review. *J. Ind. Microbiol.* 17, 170–178.
- Torsvik, V., Daae, F.L., Sandaa, R.-A., Ovreas, L., 1998. Review article: novel techniques for analysing microbial diversity in natural and perturbed environments. *J. Biotechnol.* 64, 53–62.
- Trevors, J.T., 1998a. Molecular evolution in bacteria: cell division. *Rev. Microbiol.* 29, 237–245.
- Trevors, J.T., 1998b. Bacterial biodiversity in soil with an emphasis on chemically-contaminated soils. *Water Air Soil Pollut.* 101, 45–67.
- van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396, 69–72.
- van Elsas, J.D., Wolters, A., 1995. Polymerase chain reaction (PCR) analysis of soil microbial DNA. In: Akkermans, A.D.L., van-Elsas, J.D., de-Brujin, F.J. (Eds.), *Molecular Microbial Ecology Manual*. Kluwer Academic Publishing, Boston, pp. 2.7.2 1–2.7.2 10.
- van Elsas, J.D., Frois-Duarte, G., Keijzer-Wolters, A., Smit, E., 2000. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *J. Microbiol. Methods* 43, 133–151.
- Voordouw, G., Voordouw, J.K., Karkhoff-Schweizer, R.R., Fedorak, P.M., Westlake, D.W.S., 1991. Reverse sample genome probing, a new technique for identification of bacteria in environmental samples by DNA hybridization, and its application to the identification of sulfate-reducing bacteria in oil field samples. *Appl. Environ. Microbiol.* 57, 3070–3078.
- Voordouw, G., Voordouw, J.K., Jack, T.R., Foght, J., Fedorak, P.M., Westlake, D.W.S., 1992. Identification of distinct communities of sulfate-reducing bacteria in oil fields by reverse sample genome probing. *Appl. Environ. Microbiol.* 58, 3542–3552.
- Voordouw, G., Shen, Y., Harrington, C.S., Telang, A.J., Jack, T.R., Westlake, D.W.S., 1993. Quantitative reverse sample genome probing of microbial communities and its application to oil field production waters. *Appl. Environ. Microbiol.* 59, 4101–4114.
- Wall, D.H., Virginia, R.A., 1999. Controls on soil biodiversity: insights from extreme environments. *Appl. Soil Ecol.* 13, 137–150.
- Whiteley, A.S., Bailey, M.J., 2000. Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Appl. Environ. Microbiol.* 66, 2400–2407.
- Wintzingerode, F.v., Gobel, U.B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21, 213–229.
- Wright, S.F., Upadhyaya, A., 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant Soil* 198, 97–107.
- Yao, H., He, Z., Wilson, M.J., Campbell, C.D., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. *Microb. Ecol.* 40, 223–237.
- Yu, Z., Mohn, W.W., 2001. Bioaugmentation with resin-acid-degrading bacteria enhances resin acid removal in sequencing batch reactors treating pulp mill effluents. *Water Res.* 35, 883–890.
- Zak, J.C., Willig, M.R., Moorhead, D.L., Wildman, H.G., 1994. Accelerated paper: functional diversity of microbial communities: a quantitative approach. *Soil Biol. Biochem.* 26, 1101–1108.
- Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol. Fertil. Soils* 29, 111–129.
- Zeze, A., Hosny, M., Gianinazzi-Pearson, V., Dulieu, H., 1996. Characterization of a highly repeated DNA sequence (SC1) from the arbuscular mycorrhizal fungus *Scutellospora castanea* and its detection in planta. *Appl. Environ. Microbiol.* 62, 248–2443.
- Zumstein, E., Moletta, R., Godon, J.-J., 2000. Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-strand conformation polymorphism analysis. *Environ. Microbiol.* 2, 69–78.